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IMPORTANCE OF EXTRACELLULAR PROCESSES IN THE OXYGEN ENHANCEMENT--ETC(U)
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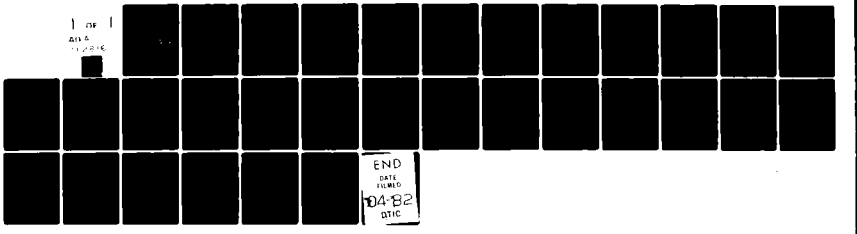

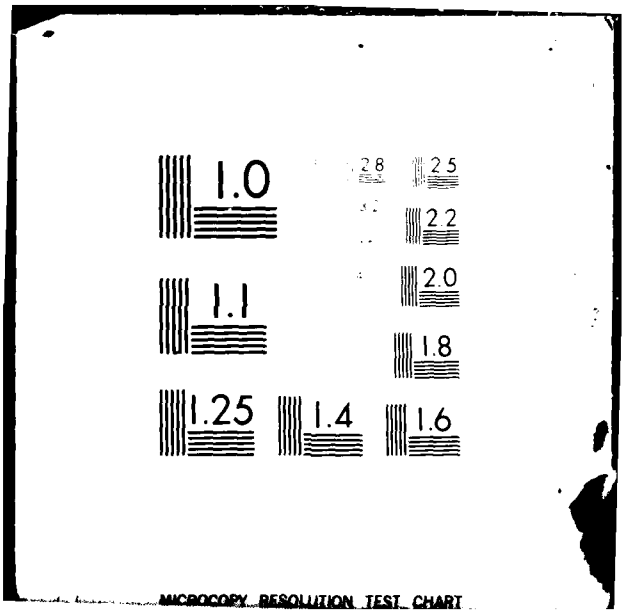
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superoxide radical	oxygen toxicity
superoxide dismutases	methyl viologen
hydroxyl radical	pyocyanine
Lactobacillus plantarum	manganese
Streptococcus sanguis	gene transfer

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We have explored the consequences of the biological production of O_2^- and of the enzymic scavenging of this radical by superoxide dismutases, and the scavenging of its dismutation product, H_2O_2, by catalases. We have shown that increased intracellular production of O_2^{-2} can kill E. coli and that elevated superoxide dismutase protects. We have isolated two hydroperoxidases from E. coli and found one to be an active catalase, while the other was both a catalase and a dianisidine peroxidase. The mechanism of oxidation of dianisidine by peroxidase was explored. (over)

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20. (continued)

A number of compounds, including methyl viologen and pyocyanine, were found to divert intracellular electron flow and to increase O_2^- production. E. coli responds adaptively to such compounds by making more superoxide dismutase. Such compounds are damaging only in the combined presence of O_2 and an electron source, because only then can they mediate O_2^- production. In L. plantarum we have found that high intracellular Mn(II), ~25 mM, provides a functional replacement for superoxide dismutase. We have also discovered the first case of a natural gene transfer between a eukaryote (ponyfish) and a prokaryote (P. leiognathi). The gene for copper-zinc superoxide dismutase appears to have been passed from the host fish to its symbiotic bacterium.

Oxygen toxicity and O_2^- toxicity has been explored in a bacterium which contains no iron compounds. In this organism (Streptococcus sanguis) we have shown that O_2^- exerts a deleterious effect which is prevented by superoxide dismutase, but not by scavengers of OH·. We conclude that critical targets for O_2^- do exist in these cells and that O_2^- can exert a lethal effect without conversion to OH·, by the iron-catalyzed Haber-Weiss process.

IMPORTANCE OF EXTRACELLULAR PROCESSES IN THE OXYGEN ENHANCEMENT OF RADIATION LETHALITY

Final Report

Irwin Fridovich
Department of Biochemistry
Duke University Medical Center
Durham, North Carolina 27710

April 15, 1978 - December 31, 1981

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3. Table of Contents

4. List of Appendixes - Abstracts

Superoxide Radicals and the Oxygen Enhancement of the Toxicity of Paraquat in Escherichia coli. H. M. Hassan and I. Fridovich

Bovine Erythrocyte Superoxide Dismutase: Diazo Coupling, Subunit Interactions and Electrophoretic Variants. D. P. Malinowski and I. Fridovich

Superoxide, Hydrogen Peroxide and Oxygen Tolerance of Oxygen-Sensitive Mutants of Escherichia coli. H. M. Hassan and I. Fridovich

Manganese-Containing Superoxide Dismutase from Escherichia coli: Reversible Resolution and Metal Replacements. D. E. Ose and I. Fridovich

Chemical and Enzymatic Intermediates in the Peroxidation of o-dianisidine by Horseradish Peroxidase. Spectral Properties of the Products of Dianisidine Oxidation. A. Claiborne and I. Fridovich

Chemical and Enzymatic Intermediates in the Peroxidation of o-dianisidine by Horseradish Peroxidase. 2. Evidence for a Substrate Radical-Enzyme Complex and Its Reaction with Nucleophiles. A. Claiborne and I. Fridovich

Purification of the o-dianisidine Peroxidase from Escherichia coli B. Physicochemical Characterization and Analysis of Its Dual Catalatic and Peroxidatic Activities. A. Claiborne and I. Fridovich.

Intracellular Production of Superoxide Radical and of Hydrogen Peroxide by Redox Active Compounds. H. M. Hassan and I. Fridovich

Paraquat and Escherichia coli: Mechanism of Production of Extracellular Superoxide Radical. H. M. Hassan and I. Fridovich

Purification and Characterization of Hydroperoxidase II of Escherichia coli B. A. Claiborne, D. P. Malinowski and I. Fridovich

Autoinactivation of Xanthine Oxidase: the Role of Superoxide Radical and Hydrogen Peroxide. R. E. Lynch and I. Fridovich

Subunit Association and Side Chain Reactivities of Bovine Erythrocyte Superoxide Dismutase in Denaturing Solvents. D. P. Malinowski and I. Fridovich

Chemical Modification of Arginine at the Active Site of the Bovine Erythrocyte Superoxide Dismutase. D. P. Malinowski and I. Fridovich

Inhibitors of Superoxide Dismutases: A Cautionary Tale. H. M. Hassan, H. Dougherty, and I. Fridovich

Mechanism of the Antibiotic Action of Pyocyanine. H. M. Hassan and I. Fridovich.

Distinguishing Between Mn Containing and Fe Containing Superoxide Dismutases in Crude Extracts of Cells. T. Kirby, J. Blum, I. Kahane, and I. Fridovich.

Putative Superoxide Dismutase Activity of Iron-EDTA: A Reexamination. J. DiGuiseppi and I. Fridovich.

Does Copper-d-Penicillamine Catalyze the Dismutation of O_2^-? P. Robertson, Jr., and I. Fridovich

Continuous Colorimetric Monitoring of the Fructose Bisphosphate Aldolase Reaction. P. Robertson, Jr., and I. Fridovich.

Ethylene from 2-keto-4-thiomethyl Butyric Acid: The Haber-Weiss Reaction. J. DiGuiseppi and I. Fridovich.

Manganese and Defenses Against Oxygen Toxicity in Lactobacillus plantarum. F. S. Archibald and I. Fridovich.

Superoxide Dismutases: Detoxication of a Free Radical. H. M. Hassan and I. Fridovich.

Superoxide Radical and Superoxide Dismutases: Threat and Defense. K. Brawn and I. Fridovich

A Reaction of the Superoxide Radical with Tetrapyrroles. P. Robertson, Jr., and I. Fridovich

Cyanide Catalyzes the Oxidation of α-Hydroxyaldehydes and Related Compounds: Monitored as the Reduction of Dioxygen, Cytochrome c and Nitroblue Tetrazolium. P. Robertson, Jr., S. E. Fridovich, H. P. Misra, and I. Fridovich.

DNA Strand Scission by Enzymically Generated Oxygen Radicals. K. Brawn and I. Fridovich

Superoxide Dismutases: Defence Against Endogenous Superoxide Radical. I. Fridovich

Superoxide Dismuting Activity of an Iron Porphyrin. Y. Ilan, I. Fridovich, R. F. Pasternak, and J.Rabani

Evidence for a Natural Gene Transfer from the Ponyfish to Its Bioluminescent Bacterial Symbiont, Photobacter leiognathi.... J. Martin and I. Fridovich

Manganese, Superoxide Dismutase and Oxygen Tolerance in Some Lactic Acid Bacteria. F. S. Archibald and I. Fridovich.

Isolation and Characterization of the Iron-Containing Superoxide Dismutase of Methanobacterium bryantii. T. W. Kirby, J. R. Lancaster, Jr. and I. Fridovich

The Scavenging of Superoxide Radical by Manganous Complexes, in vitro. F. S. Archibald and I. Fridovich

Investigations of the State of the Manganese in Lactobacillus plantarum. F. S. Archibald and I. Fridovich

Superoxide Radical Inhibits Catalase. Y. Kono and I. Fridovich

Oxygen Toxicity in Streptococcus sanguis. The Relative Importance of Superoxide and of Hydroxyl Radicals. J. DiGuiseppi and I. Fridovich

5. Body of Report

a. Problem Studied - Having discovered that superoxide radicals are regularly generated in living systems and are catalytically scavenged by specific defensive enzymes (superoxide dismutases), we have devoted ourselves to studying the effects of O_2^- on cells and the nature, distribution, mechanisms and biological functions of the superoxide dismutases.

b. Summary of the Most Important Results - We have explored the consequences of the biological production of O_2^- and of the enzymic scavenging of this radical by superoxide dismutases, and the scavenging of its dismutation product, H_2O_2, by catalases. We have shown that increased intracellular production of O_2^- can kill E. coli and that elevated superoxide dismutases protects. We have isolated two hydroperoxidases from E. coli and found one to be an active catalase, while the other was both a catalase and a dianisidine peroxidase. The mechanism of oxidation of dianisidine by peroxidase was explored.

A number of compounds, including methyl viologen and pyocyanine, were found to divert intracellular electron flow and to increase O_2^- production. E. coli responds adaptively to such compounds by making more superoxide dismutase. Such compounds are damaging only in the combined presence of O_2 and an electron source, because only then can they mediate O_2^- production. In L. plantarum we have found that high intracellular Mn(II), ~25 mM, provides a functional replacement for superoxide dismutase. We have also discovered the first case of a natural gene transfer between a eukaryote (ponyfish) and a prokaryote (P. leiognathi). The gene for copper-zinc superoxide dismutase appears to have been passed from the host fish to its symbiotic bacterium.

Oxygen toxicity and O_2^- toxicity have been explored in a bacterium which contains no iron compounds. In this organism (Streptococcus sanguis) we have shown that O_2^- exerts a deleterious effect which is prevented by superoxide dismutase, but not by scavengers of $OH\cdot$. We conclude that critical targets for O_2^- do exist in these cells and that O_2^- can exert a lethal effect without conversion to $OH\cdot$, by the iron-catalyzed Haber-Weiss process.

5c. Publications

4/15/78-12/31/81

H. M. Hassan and I. Fridovich, Superoxide radicals and the oxygen enhancement of the toxicity of paraquat in Escherichia coli. J. Biol. Chem. 253, 8143-8148 (1978).

D. P. Malinowski and I. Fridovich, Bovine erythorcyte superoxide dismutase: diazo coupling, subunit interactions and electrophoretic variants. Biochemistry 18, 237-244 (1979).

H. M. Hassan and I. Fridovich, Superoxide, hydrogen peroxide and oxygen tolerance of oxygen-sensitive mutants of Escherichia coli. Rev. Infect. Dis. 1, 357-369 (1979).

D. E. Ose and I. Fridovich, Manganese-containing superoxide dismutase from Escherichia coli: reversible resolution and metal replacements. Arch. Biochem. Biophys. 194, 360-364 (1979).

A. Claiborne and I. Fridovich, Chemical and enzymatic intermediates in the peroxidation of o-dianisidine by horseradish peroxidase. 1. Spectral properties of the products of dianisidine oxidation. Biochemistry 18, 2324-2329 (1979).

A. Claiborne and I. Fridovich, Chemical and enzymatic intermediates in the peroxidation of o-dianisidine by horseradish peroxidase. 2. Evidence for a substrate radical-enzyme complex and its reaction with nucleophiles. Biochemistry 18, 2329-2335 (1979).

A. Claiborne and I. Fridovich, Purification of the o-dianisidine peroxidase from Escherichia coli B. Physicochemical characterization and analysis of its dual catalatic and peroxidatic activities. J. Biol. Chem. 254, 4245-4252 (1979).

H. M. Hassan and I. Fridovich, Intracellular production of superoxide radical and of hydrogen peroxide by redox active compounds. Arch. Biochem. Biophys. 196, 385-395 (1979).

H. M. Hassan and I. Fridovich, Paraquat and Escherichia coli: Mechanism of production of extracellular superoxide radical. J. Biol. Chem. 254, 10846-10852 (1979).

A. Claiborne, D. P. Malinowski and I. Fridovich, Purification and characterization of hydroperoxidase II of Escherichia coli B. J. Biol. Chem. 254, 11664-11668 (1979).

R. E. Lynch and I. Fridovich, Autoinactivation of xanthine oxidase: the role of superoxide radical and hydrogen peroxide. Biochim. Biophys. Acta 571, 195-200 (1979).

D. P. Malinowski and I. Fridovich, Subunit association and side chain reactivities of bovine erythrocyte superoxide dismutase in denaturing solvents. Biochemistry 18, 5055-5060 (1979).

D. P. Malinowski and I. Fridovich, Chemical modification of arginine at the active site of the bovine erythrocyte superoxide dismutase. Biochemistry 18, 5909-5917 (1979).

H. M. Hassan, H. Dougherty, and I. Fridovich, Inhibitors of superoxide dismutases: a cautionary tale. Arch. Biochem. Biophys. 199, 349-354 (1980).

H. Moustafa Hassan and I. Fridovich, Mechanism of the antibiotic action of pyocyanine. J. Bacteriol. 141, 156-163 (1980).

T. Kirby, J. Blum, I. Kahane, and I. Fridovich, Distinguishing between Mn containing and Fe containing superoxide dismutases in crude extracts of cells. Arch. Biochem. Biophys. 201, 551-555 (1980).

J. DiGuiseppi and I. Fridovich, Putative superoxide dismutase activity of iron-EDTA: A reexamination. Arch. Biochem. Biophys. 203, 145-150 (1980).

P. Robertson, Jr., and I. Fridovich, Does copper-d-penicillamine catalyze the dismutation of O_2^-? Arch. Biochem. Biophys. 203, 830-831 (1980).

P. Robertson, Jr., and I. Fridovich, Continuous colorimetric monitoring of the fructose bisphosphate aldolase reaction. Anal. Biochem. 108, 332-334 (1980).

J. DiGuiseppi and I. Fridovich, Ethylene from 2-keto-4-thiomethyl butyric acid: the Haber-Weiss reaction. Arch. Biochem. Biophys. 205, 323-329 (1980).

K. Brawn and I. Fridovich, Superoxide radical and superoxide dismutases: threat and defense. Acta Physiol. Scand. Suppl. 492, 9-18 (1980).

H. M. Hassan and I. Fridovich, Superoxide dismutases: detoxication of a free radical. In Enzymatic Basis of Detoxication, Vol. I (W. B. Jacoby, ed.), Academic Press, New York, 1980, p. 311.

F. S. Archibald and I. Fridovich, Manganese and defenses against oxygen toxicity in Lactobacillus plantarum. J. Bacteriol. 145, 442-451 (1981).

K. Brawn and I. Fridovich, DNA strand scission by enzymically generated oxygen radicals. Arch. Biochem. Biophys. 206, 414-419 (1981).

P. Robertson, Jr., S. E. Fridovich, H. P. Misra, and I. Fridovich, Cyanide catalyzes the oxidation of α-hydroxyaldehydes and related compounds: monitored as the reduction of dioxygen, cytochrome c, and nitroblue tetrazolium. Arch. Biochem. Biophys. 207, 282-289 (1981).

I. Fridovich, Superoxide dismutases: defense against endogenous superoxide radical. In Ciba Foundation Series 65, Excerpta Medica, Elsevier North-Holland, pp. 77-93, 1979.

Y. Ilan, I. Fridovich, R. F. Pasternak, and J. Rabani, Superoxide dismuting activity of an iron porphyrin. Inorg. Nucl. Chem. Lett. 17, 93 (1981).

J. Martin and I. Fridovich, Evidence for a natural gene transfer from the ponyfish to its bioluminescent bacterial symbiont, Photobacter leiognathi: the close relationship between bacteriocuprein and the copper zinc superoxide dismutase of teleost fishes. J. Biol. Chem. 256, 6080-6089 (1981).

T. W. Kirby, J. R. Lancaster, Jr., and I. Fridovich, Isolation and characterization of the iron-containing superoxide dismutase of Methanobacterium bryantii. Arch. Biochem. Biophys. 210, 140-148 (1981).

I. Fridovich, Superoxide radical and superoxide dismutases. In Oxygen and Living Processes (D. L. Gilbert, ed.) Springer-Verlag, New York, 1981, pp. 250-272.

F. S. Archibald and I. Fridovich, Manganese, superoxide dismutase and oxygen tolerance in some lactic acid bacteria. J. Bacteriol. 146, 928-936 (1981).

P. Robertson, Jr., and I. Fridovich, A reaction of the superoxide radical with tetrapyrroles. Arch. Biochem. Biophys. 213, 353-357 (1982).

F. S. Archibald and I. Fridovich, The scavenging of superoxide radical by manganous complexes, in vitro. Arch. Biochem. Biophys., in press.

F. S. Archibald and I. Fridovich, Investigations of the state of the manganese in Lactobacillus plantarum, Arch. Biochem. Biophys., in press.

Y. Kono and I. Fridovich, Superoxide radical inhibits catalase. J. Biol. Chem., in press.

J. DiGuiseppi and I. Fridovich, Oxygen toxicity in Streptococcus sanguis. The relative importance of superoxide and of hydroxyl radicals. J. Biol. Chem., in press (1982).

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Douglas P. Malinowski, earned Ph.D.

Marvin Salin, Postdoctoral Fellow
Joseph P. Martin, Postdoctoral Fellow
Karl Beem, Postdoctoral Fellow
Amelia Cudd, Postdoctoral Fellow
Y. Kono, Postdoctoral Fellow
Haim Rabinowitch, Postdoctoral Fellow

H. M. Hassan and I. Fridovich, Superoxide radical and the oxygen enhancement of the toxicity of paraquat in Escherichia coli. J. Biol. Chem. 253, 8143-8148.

Paraquat (methyl viologen) was much more toxic to *Escherichia coli* in the presence of oxygen than in its absence and, in the presence of oxygen, was more toxic in a simple nutritionally restricted medium than it was in a rich complex medium. Yeast extract, added to the simple glucose-salts medium, minimized the toxicity of aerobic paraquat and also facilitated maximal induction of superoxide dismutase. Preventing this induction of superoxide dismutase with an inhibitor of protein synthesis, such as puromycin, greatly augmented the toxicity of paraquat. Many of the cells, which survived exposure to aerobic paraquat in the presence of puromycin, gave rise to microcolonies on trypticase/soy/yeast extract medium-agar reflecting sublethal damage. The lethality of paraquat, in the presence of puromycin, was entirely dependent upon the presence of oxygen, but 0.2 atm of O_2 was already a saturating amount and a 100-fold increase in pO_2 did not cause a greater effect. Such was the oxygen sensitivity of cells exposed to paraquat, in the presence of puromycin, that exposure to air after anaerobic dilution and plating, resulted in 96% mortality. An abundant supply of electrons was also essential for the expression of paraquat toxicity. Thus, cells exposed in the absence of nutrients, or in the presence of nutrients to which they were not adapted, suffered minimal lethality. Cells containing high intracellular levels of superoxide dismutase, because of growth on inducing media or because of prior induction with pyocyanine, were resistant toward the lethality of aerobic paraquat.

It may be concluded that paraquat is toxic and lethal to *E. coli* largely because it can be reduced within the cell and then reoxidized by molecular oxygen, with the production of O_2^-. Paraquat toxicity is thus largely a superoxide toxicity and it is diminished by superoxide dismutase.

VOL. 18, NO. 1, 1979 237 - 244

Bovine Erythrocyte Superoxide Dismutase: Diazo Coupling, Subunit Interactions, and Electrophoretic Variants†

Douglas P. Malinowski and Irwin Fridovich*

ABSTRACT: The copper and zinc-containing superoxide dismutase of bovine erythrocytes was modified and inactivated by several diazonium reagents. Treatment of native or of zinc-only enzymes, with diazonium 1*H*-tetrazole, derivatized 1 tyrosine, 1 histidine, and 10 lysine residues per subunit. The partial inactivation, accompanying this treatment, appeared to be due to modification of the lysine residues, since acetylation caused a similar extent of lysine modification and of inactivation. Apoenzyme exhibited greater reactivity toward diazonium 1*H*-tetrazole, reflecting the exposure of two histidine residues per subunit in the active site region. Diazo coupling with diazonium 1*H*-tetrazole grossly increased the anodic mobility of the enzyme and yielded a derivative useful for studies of subunit interactions. Admixture of native enzyme with diazo-coupled, H_2O_2-inactivated enzyme, in the presence of 8.0 M urea, followed by removal of the urea, generated a new, enzymatically active, species. The new species exhibited an electrophoretic mobility nearly intermediate between that of the native and diazo-coupled proteins, but had the same molecular weight as the parental species. When isolated from polyacrylamide gels and incubated at pH 7.8 for 2 h at 25 °C, the new species gave rise to both parental species, demonstrating that it was an unstable hybrid. The specific activity of the hybrid was approximately half that of the native enzyme. A native subunit thus exhibited the same catalytic activity, whether paired with another native subunit or with a chemically modified and catalytically inactive subunit. Mutually inhibitory interactions between subunits, giving rise to half of the sites reactivity, thus seems unlikely.

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Superoxide, Hydrogen Peroxide, and Oxygen Tolerance of Oxygen-Sensitive Mutants of *Escherichia coli*

H. Moustafa Hassan and Irwin Fridovich

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

Oxygen-intolerant mutants of *Escherichia coli* K12 were selected by a replica plating technique after treatment with the mutagen, N-methyl-N'-nitro-N-nitrosoguanidine, to a lethality of 99.5%. One group of mutants had lost the ability to induce both peroxidase and catalase when exposed to oxygen but retained the ability to induce the manganese-superoxide dismutase. The second group of mutants had lost the ability to induce the activity of all these enzymes. Failure to induce peroxidase and catalase was associated with enhanced susceptibility of the bacteria to the lethal effect of oxygen. When a member of the first group of mutants was prevented from producing the manganese-superoxide dismutase by the presence of puromycin, its susceptibility to the lethal effects of oxygen was greatly increased. Two types of revertants were seen. In one group the ability to induce enzyme activity was recovered and was accompanied by the return of oxygen tolerance. Members of the other group lost the ability to respire and, therefore, no longer produced O_2^- and H_2O_2. These results indicated that enzymic scavenging of both H_2O_2 and O_2^- provides an important defense against oxygen toxicity. The parallel loss of peroxidase and catalase, which was seen in all mutants, suggests that these enzymes constitute a precursor-product pair in *E. coli*. The parallel loss in two of these mutants of peroxidase, catalase, and the manganese-superoxide dismutase suggests a control linkage for these enzymes, the basis of which remains to be explored.

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Manganese-Containing Superoxide Dismutase from *Escherichia coli*: Reversible Resolution and Metal Replacements[1]

DENNIS E. OSE AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received September 11, 1978; revised December 11, 1978

Exposure of the manganese-containing superoxide dismutase of *Escherichia coli* to pH 3.2, in the presence of 0.7 M guanidinium chloride, causes a rapid loss of manganese and of activity. The apoenzyme so produced can be reconstituted by addition of $MnCl_2$ followed by neutralization. In contrast, manganese cannot be restored to the apoenzyme by adding $MnCl_2$ *after* neutralization. The reconstituted enzyme is indistinguishable from the native enzyme in terms of its catalytic activity or electrophoretic behavior on polyacrylamide gels. Co(II), Ni(II), Zn(II), Fe(II), or Cu(II) could compete with Mn(II) during reconstitution of the apoenzyme. In the cases of Co(II), Ni(II), and Zn(II), it was shown that, in preventing reconstitution by Mn(II), they were themselves bound to the enzyme in stoichiometric amounts, in place of Mn(II). The binding of Fe(II) was also explored and was distinct in that the enzyme could bind more than stoichiometric amounts of this metal. None of the derivatives, in which Mn(II) had been replaced by another metal, were catalytically active. Nevertheless, these derivatives could be again resolved by exposure to acid guanidinium chloride and could then be converted back into the active holoenzyme by neutralization after addition of $MnCl_2$. It appears that the active site of this enzyme can accommodate and can tightly bind several metals other than manganese, but exhibits activity only with manganese. It also appears that movement of metal out of or into this site is only feasible at low pH and in the presence of a chaotropic agent. A substantial amount of the cobalt-substituted enzyme was prepared and its optical properties were recorded.

Chemical and Enzymatic Intermediates in the Peroxidation of *o*-Dianisidine by Horseradish Peroxidase. 1. Spectral Properties of the Products of Dianisidine Oxidation†

Al Claiborne and Irwin Fridovich*

Biochemistry 18, 2324-2329 (1979).

ABSTRACT: Studies of the optical spectra of the products formed during peroxidation of *o*-dianisidine by horseradish peroxidase indicate at least three distinct species. At pH 3.7 and 4 °C, peroxidation of dianisidine at low concentrations yields the free dianisidine quinonediimine (the two-equivalent oxidized form) with λ_{max} 452 and 514 nm. At higher concentrations, the first detectable product is not the free quinonediimine but an intermolecular complex (meriquinone or charge-transfer complex) consisting of quinonediimine and parental diamine. This complex is freely reversible and is sensitive to simple dilution or acidification, either of which restores the spectrum of the free quinonediimine. Furthermore, at near-neutral pH, the quinonediimine appears to undergo irreversible self-coupling, yielding yet a different optical spectrum presumably characteristic of the bisazobiphenyl structure proposed by K. M. Møller & P. Ottolenghi [(1966) *C. R. Trav. Lab. Carlsberg 35*, 369–389]. Butylated hydroxyanisole was shown to react in the presence of peroxidase–H_2O_2 and dianisidine to yield a spectrum (λ_{max} 575 nm) nearly identical with that obtained when Gibbs' reagent (2,6-dichloroquinone 4-chloroimine) was incubated with butylated hydroxyanisole, thus suggesting that the free quinonediimine itself couples with the phenolic antioxidant. Finally, continuous-flow EPR studies of dianisidine oxidation both with HRP–H_2O_2 and with ceric sulfate were unable to detect any free dianisidine semiquinone radical in the steady state; we conclude that oxidation of dianisidine occurs in a rapid two-electron process in both the HRP–H_2O_2 and Ce(IV) systems.

Chemical and Enzymatic Intermediates in the Peroxidation of *o*-Dianisidine by Horseradish Peroxidase. 2. Evidence for a Substrate Radical–Enzyme Complex and Its Reaction with Nucleophiles†

Biochemistry 18, 2329-2335 (1979).

Al Claiborne and Irwin Fridovich*

ABSTRACT: Changes in the optical absorption spectrum of horseradish peroxidase, during the oxidation of *o*-dianisidine at pH 7.5, reveal an intermediate distinct from the previously described compounds I and II. The rate of decay of this new complex appeared to be rate limiting for the catalytic cycle, in this pH range, since imidazole, which augments the catalytic reaction, also enhanced the rate of decay of this complex. Nitrogenous compounds reportedly unable to ligate to hemes, such as 2-methylimidazole and benzimidazole, were nevertheless capable of augmenting the HRP-catalyzed rate of oxidation of *o*-dianisidine. The activity of nitrogenous compounds, in this regard, appeared to be a function of their nucleophilicity and was sensitive to steric factors but relatively free of a deuterium solvent isotope effect. The data presented in this and in the preceding paper [Claiborne, A., & Fridovich, I. (1979) *Biochemistry 18* (preceding paper in this issue)] lead to the suggestion that the nucleophile-responsive intermediate is an enzyme–dianisidine radical complex and that abstraction of the second electron from the bound radical is facilitated by binding of nitrogenous nucleophiles.

A. Claiborne and I. Fridovich, Purification of the o-Dianisidine Peroxidase from Escherichia coli B. Physicochemical Characterization and Analysis of Its Dual Catalytic and Peroxidatic Activities. J. Biol. Chem. 254, 4245-4252 (1979).

Extracts of aerobically grown *Escherichia coli* B exhibit both catalase and dianisidine peroxidase activities. Polyacrylamide gel electrophoresis demonstrates two distinct catalases which have been designated hydroperoxidases I and II (HP-I and HP-II) in order of increasing anodic mobility. HP-I has been purified to essential homogeneity and found to be composed of four subunits of equal size. Its molecular weight is 337,000, and it contains two molecules of protoheme IX per tetramer. Its amino acid composition is unusual, for so large a protein, in lacking half-cystine. HP-I is a very efficient catalase with an activity optimum at pH 7.5, a K_m for H_2O_2 of 3.9 mM, and a turnover number of 9.8×10^5 per min. It is also a broad specificity peroxidase capable of acting upon dianisidine, guaiacol, *p*-phenylenediamine, and pyrogallol. Dianisidine acted as a powerful reversible inhibitor of the catalatic activity of HP-I and as a suicide substrate when HP-I functioned in its peroxidatic mode.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 196, No. 2, September, pp. 385–395, 1979

Intracellular Production of Superoxide Radical and of Hydrogen Peroxide by Redox Active Compounds[1]

H. MOUSTAFA HASSAN AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received March 5, 1979; revised May 2, 1979

Several compounds have been found capable of diverting the electron flow in *Escherichia coli* and thus causing increased intracellular production of O_2^- and H_2O_2. One indication of this electron-shunting action was increased cyanide-resistant respiration and one cellular response was increased biosynthesis of the manganese-containing superoxide dismutase and of catalase. Blocking cytochrome oxidase with cyanide or azide increased the electron flow available for reduction of paraquat and presumably of the other exogenous compounds tested and thus increased their biological effects. Paraquat, pyocyanine, phenazine methosulfate, streptomycin, juglone, menadione, plumbagin, methylene blue, and azure C were all effective in elevating intracellular production of O_2^- and H_2O_2. The effect of alloxan appeared paradoxical in that it increased cyanide-resistant respiration without significantly increasing the cell content of the manganese-superoxide dismutase and with only a small effect on the level of catalase. The alloxan effect on cyanide-resistant respiration was artifactual and was due to an oxygen-consuming reaction between alloxan and cyanide, rather than to a diversion of the intracellular electron flow. With paraquat as a representative electron-shunting compound, the increase in biosynthesis of the manganese-superoxide dismutase was prevented by inhibitors of transcription or of translation, but not by an inhibitor of replication. The increase in this enzyme activity, caused by paraquat and presumably by the other compounds, was thus due to *de novo* enzyme synthesis activated or derepressed at the level of transcription.

H. M. Hassan and I. Fridovich, Paraquat and E. coli. Mechanism of Production of Extracellular Superoxide Radical. J. Biol. Chem. 254, 10846-10852 (1979).

Paraquat mediates a superoxide dismutase-inhibitable reduction of cytochrome *c* by suspensions of *Escherichia coli* B. Glucose was most effective in providing electrons for this cytochrome *c* reduction, but other nutrients could serve in this capacity, provided the cells were preconditioned by growth on these nutrients. Paraquat reduction depended upon a NADPH:paraquat diaphorase, present in the cytosol. Reduced paraquat could diffuse across the cell envelope and react with dioxygen, in the suspending medium, thus generating O_2^- in that compartment. Most of the paraquat reduced in the cell, under the conditions used, reoxidized *in situ* and most of the O_2^- production was thus intracellular. The partitioning of reduced paraquat between intracellular and extracellular compartments, prior to reaction with dioxygen, depended upon intracellular pO_2 and any strategy which raised intracellular pO_2 decreased the efflux of reduced paraquat and thus decreased extracellular O_2^- production. Extracellular O_2^- and H_2O_2 did contribute to cell damage in proportion to the amount produced. O_2^- appeared to be unable to cross the cell envelope in either direction and the only O_2^- which was effective in raising the rate of biosynthesis of the manganese-superoxide dismutase, was that generated within the cell.

A. Claiborne, D. P. Malinowski and I. Fridovich, Purification and Characterization of Hydroperoxidase II of E. coli B. J. Biol. Chem. 254, 11664-11668 (1979).

The second heme-containing hydroperoxidase isozyme (HP-II) has been isolated from aerobic cultures of *Escherichia coli* B. The protein exists as a stable tetramer of subunits of equal size, with a combined molecular weight of 312,000. The heme spectrum of HP-II is unusual, in that it exhibits two absorbance maxima at 407 and 591 nm; the alkaline pyridine hemochromogen spectrum shows maxima at 425, 559, and 609 nm. HP-II differs in several respects from the HP-I isozyme previously reported (Claiborne, A., and Fridovich, I. (1979) *J. Biol. Chem.* 254, 4245-4252). Thus HP-II is virtually devoid of peroxidatic activity toward *o*-dianisidine but has a 6-fold higher catalatic activity than HP-I. Antisera to HP-II do not cross-react with HP-I, and analyses of chymotryptic and cyanogen bromide digests suggest differences in primary structure between these two isozymes.

Biochimica et Biophysica Acta, 571 (1979) 195—200

BBA 68860

AUTOINACTIVATION OF XANTHINE OXIDASE

THE ROLE OF SUPEROXIDE RADICAL AND HYDROGEN PEROXIDE

ROBERT E. LYNCH * and IRWIN FRIDOVICH

Division of Hematology-Oncology, University of Utah Medical Center, Salt Lake City, UT 84132, and Department of Biochemistry, Duke University Medical Center, Durham, NC 27710 (U.S.A.)

(Received March 23rd, 1979)

Key words: Xanthine oxidase autoinactivation; Superoxide radical; Hydrogen peroxide

Summary

Xanthine oxidase suffers autoinactivation in the course of catalyzing the oxidation of acetaldehyde. When no special efforts were made to maintain a high pO_2 in these reaction mixtures catalase protected the xanthine oxidase, but superoxide dismutase did not. However, when oxygen depletion was slowed or prevented by working at lower concentrations of xanthine oxidase, at lower temperatures or by vigorous agitation under an atmosphere of 100% oxygen, superoxide dismutase or catalase protected markedly when added separately and protected almost completely when added together. This result correlates with the greater production of O_2^-, relative to H_2O_2, by xanthine oxidase, at elevated pO_2. Since histidine also provided some protection and the high levels of acetaldehyde used would have precluded any significant effect of $OH^\cdot$, we conclude that singlet oxygen, or something with similar reactivity, was generated from O_2^- plus H_2O_2 and contributed significantly to the observed autoinactivation.

[Reprinted from Biochemistry, (1979) 18, 5055.]

Subunit Association and Side-Chain Reactivities of Bovine Erythrocyte Superoxide Dismutase in Denaturing Solvents†

Douglas P. Malinowski and Irwin Fridovich*

ABSTRACT: The copper- and zinc-containing superoxide dismutase of bovine erythrocytes retains its native molecular weight of 32 000 in 8.0 M urea for at least 72 h at 25 °C, as evidenced by sedimentation equilibrium analysis. Subsequent to prolonged exposure to urea, the dimeric enzyme could be dissociated by sodium dodecyl sulfate in the absence of reductants, indicating the absence of unnatural disulfide cross-links. The sulfhydryl group of cysteine-6 was unreactive toward 5,5′-dithiobis(2-nitrobenzoic acid) or bromoacetic acid in both neutral buffer and 8.0 M urea. The histidine residues of the enzyme were resistant to carboxymethylation in neutral buffer and 8.0 M urea. However, when the enzyme was exposed to bromoacetic acid in the presence of 6.0 M guanidinium chloride and 1 mM (ethylenedinitrilo)tetraacetic acid (EDTA), both sulfhydryl and histidine alkylation were observed. Guanidinium chloride (6.0 M) increased the reactivity of the sulfhydryl group of cysteine-6 and allowed the oxidative formation of disulfide-bridged dimers. This was prevented by 1 mM EDTA. It follows that 8.0 M urea neither dissociates the native enzyme into subunits nor produces a conformation detectably different than that possessed under native conditions.

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Chemical Modification of Arginine at the Active Site of the Bovine Erythrocyte Superoxide Dismutase†

Douglas P. Malinowski and Irwin Fridovich*

ABSTRACT: Several α,β-diketones inactivated the bovine erythrocyte superoxide dismutase, while modifying 1 arginine residue/subunit. With phenylglyoxal it was shown that the degree of inactivation was a linear function of the extent of arginine modification, to a limit of 1 arginine/subunit, and that arginine modification caused extensive changes in the visible absorbance attributed to the copper at the active site. Butanedione or cyclohexanedione plus borate causes a modification of arginine which is reversible by dialysis. With these reagents it was shown that reversal of arginine modification restored the lost activity. Since this reactivation was seen even when (ethylenedinitrilo)tetraacetic acid was present during dialysis, it follows that copper loss was not a factor in the inactivation which accompanied arginine modification. Analysis of tryptic fragments of cyanogen bromide peptides demonstrated that the essential arginine was no. 141 in the linear sequence. This residue is known, from X-ray crystallography, to lie within 6 Å of the active-site copper. Modification of arginine diminished activity to a limit of 10–20% of the native activity, as measured at pH 7.8. It appears possible that arginine may provide electrostatic attraction for incoming O_2^- or may serve in proton conduction during the second half of the catalytic cycle. Alternately, modification of this arginine may simply distort the ligand field of the Cu(II). Copper- and zinc-containing superoxide dismutases from wheat germ, chicken liver, and several mammalian sources all exhibited comparable sensitivity to arginine-modifying reagents.

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Inhibitors of Superoxide Dismutases: A Cautionary Tale†

H. MOUSTAFA HASSAN,* HARRY DOUGHERTY,‡ AND IRWIN FRIDOVICH*,²

*Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, and ‡Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065

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An extensive search resulted in the identification of pamoic acid as an inhibitor of superoxide dismutases. Pamoic acid appeared to rapidly and reversibly inhibit all types of superoxide dismutases and did so in both the cytochrome *c* reduction and in the dianisidine photooxidation assays, used to measure this activity. It could nevertheless be shown that pamoic acid did not at all inhibit superoxide dismutase but rather diminished the sensitivity of the assays. The mechanism proposed to account for this effect involved oxidation of pamoate, by O_2, to yield a pamoate radical which can then reduce cytochrome *c* or oxidize pyrogallol. Pamoate thus competes with superoxide dismutase for the available O_2, without affecting the observable effects of that O_2 upon cytochrome *c* or upon pyrogallol. It consequently makes these assays less responsive to superoxide dismutase, while appearing to be without effect in the absence of superoxide dismutase. Several of the predicted consequences of this proposal were affirmed. Other workers, interested in finding inhibitors for superoxide dismutases, are hereby forwarned of this subtle snare.

JOURNAL OF BACTERIOLOGY, Jan. 1980, p. 156–163
0021-9193/80/01-0156/08$02.00/0

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Mechanism of the Antibiotic Action of Pyocyanine

H. MOUSTAFA HASSAN† AND IRWIN FRIDOVICH*

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Exposure of *Escherichia coli* growing in a rich medium to pyocyanine resulted in increased intracellular levels of superoxide dismutase and of catalase. When these adaptive enzyme syntheses were prevented by nutritional paucity, the toxic action of pyocyanine was augmented. The antibiotic action of pyocyanine was dependent upon oxygen and was diminished by superoxide dismutase and by catalase, added to the suspending medium. Pyocyanine slightly augmented the respiration of *E. coli* suspended in a rich medium, but greatly increased the cyanide-resistant respiration. Pyocyanine was able to cause the oxidation of reduced nicotinamide adenine dinucleotide, with O_2^- production, in the absence of enzymatic catalysis. It is concluded that pyocyanine diverts electron flow and thus increases the production of O_2^- and H_2O_2 and that the antibiotic action of this pigment is largely a reflection of the toxicity of these products of oxygen reduction.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
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Distinguishing between Mn-Containing and Fe-Containing Superoxide Dismutases in Crude Extracts of Cells[1]

THOMAS KIRBY,* JANICE BLUM,*, ITZHAK KAHANE,†
AND IRWIN FRIDOVICH*,[2]

**Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 and*
†The Biomembrane Research Laboratory, The Hebrew University Hadassah Medical School, Jerusalem, Israel

Received November 30, 1979

Superoxide dismutases containing manganese or iron can be resolved by exposure to low pH in the presence of guanidinium chloride. Apoenzymes so produced are inactive and are reactivated only by the metal characteristic of the native enzymes. In crude extracts of procaryotes, which may contain the iron enzymes or the manganese enzyme or both together, one can, by resolution followed by treatment with Fe(II) or Mn(II), identify and distinguish these enzymes. This method was validated with extracts of *Escherichia coli*, known to contain both types of enzymes, and was then used to demonstrate the presence of an iron superoxide dismutase in *Alcaligenes faecalis* and of manganese superoxide dismutases in *Streptococcus sanguis*, *S. lactis*, *Bacillus megaterium*, and *Acholeplasma laidlawii*. The *A. laidlawii* enzyme was found to have a molecular weight of ~41,000.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 203, No. 1, August, pp. 145–150, 1980

Putative Superoxide Dismutase Activity of Iron–EDTA: A Reexamination[1]

JAMES DIGUISEPPI AND IRWIN FRIDOVICH[2]

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, 27710

Received January 7, 1980

It has been reported that iron–EDTA complexes mimic the action of superoxide dismutase, displaying 0.01% of the activity of the enzyme (Halliwell, B., 1975, *FEBS Lett.*, **56**, 34–38). This was purportedly directly confirmed by J. G. McClune, J. A. Fee, G. A. McCluskey, and J. T. Groves, 1977, *J. Amer. Chem. Soc.*, **99**, 5220–5222. A reexamination of the behavior of this compound has demonstrated that it does not catalyze the dismutation of O_2^-, but rather interferes with assays for superoxide dismutation activity, which are based on the reductions of nitroblue tetrazolium or of cytochrome *c*. The sources of this interference have been examined. Investigators engaged in searching for mimics of superoxide dismutase are urged to be wary of similar artifacts.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
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Does Copper-D-Penicillamine Catalyze the Dismutation of O_2^-?[1]

PHILMORE ROBERTSON, JR. AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received May 19, 1980

It has been reported (M. Younes and U. Weser, 1977, *Biochem. Biophys. Res. Commun.* **78**, 1247–1253; E. Lengfelder and E. F. Elstner, 1978, *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 751–757) that the complex $[Cu(I)_4Cu(II)_8(D\text{-penicillamine})_{12}Cl]^5$ efficiently catalyzes the dismutation of O_2^- and that this activity is resistant to both EDTA and CN^-. However, careful study has demonstrated that this complex is unable to catalyze the dismutation of O_2^-, but that it slowly decomposes to simpler copper complexes which are active. Moreover, the activity which is observed is suppressed by EDTA or by Chelex 100 treatment.

ANALYTICAL BIOCHEMISTRY **108**, 332–334 (1980)

Continuous Colorimetric Monitoring of the Fructose Bisphosphate Aldolase Reaction[1]

PHILMORE ROBERTSON, JR., AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received March 25, 1980

A simple method for continuous spectrophotometric assay of fructose-1,6-bisphosphate aldolase is described. The method is based on the reactivity of the product triose phosphates with cyanide to form compounds capable of the reduction of cytochrome *c*. At the concentrations employed, cyanide, acting catalytically, reacts with the enediol tautomer of the triose phosphates to generate the reductants, which reduce cytochrome *c* causing increased absorbance at 550 nm. The rate of increase in the rate of appearance of 550-nm absorbance is directly proportional to the concentration of aldolase present. The procedure is particularly useful for Class I aldolases, since the assay must be run under mildly alkaline conditions.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
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Ethylene from 2-Keto-4-thiomethyl Butyric Acid: The Haber–Weiss Reaction[1]

JAMES DIGUISEPPI AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received May 19, 1980

2-Keto-4-thiomethyl butyric acid is cooxidized, with production of ethylene, by the xanthine oxidase reaction. Ethylene production was inhibited by either superoxide dismutase or catalase indicating an essential role for both $O_2^{\cdot-}$ and H_2O_2. Ethylene production was dependent upon iron complexes, while a variety of other metals were found ineffective. At pH 7.8 ethylenediaminetetraacetate–iron was much more effective in facilitating ethylene production, in the xanthine oxidase system, than was diethylenetriaminepentaacetate–iron; yet these two chelates were equally effective in ethylene production dependent upon the Fenton reaction. pH was an important variable in determining the activity of iron chelates in facilitating ethylene production in the xanthine oxidase system. The effectiveness of hydroxyl radical scavengers, in preventing ethylene production, was directly related to their abilities to scavenge $OH\cdot$ and this was the case in the presence of any of the several chelating agents tested. The proximal oxidant, responsible for ethylene production, thus appears to be $OH\cdot$, rather than some metal–oxy complex.

JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 442–451
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Vol. 145, No. 1

Manganese and Defenses against Oxygen Toxicity in *Lactobacillus plantarum*

FREDERICK S. ARCHIBALD AND IRWIN FRIDOVICH*

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Lactobacillus plantarum is aerotolerant during log-phase growth on glucose, but is an obligate aerobe on polyols. Respiration was cyanide resistant and under certain conditions was associated with the accumulation of millimolar concentrations of H_2O_2. On glucose, optimal growth was observed in the absence of O_2. Extracts of *L. plantarum* did not catalyze the reduction of paraquat by reduced nicotinamide adenine dinucleotide, but plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) was readily reduced. Such extracts produced $O_2^{\cdot-}$ in the presence of NADH plus plumbagin. Plumbagin caused a 10-fold increase in the rate of respiration of intact cells in the presence of glucose and also imposed a loss of viability which was dependent upon both glucose and O_2. Although extracts of *L. plantarum* were devoid of true superoxide dismutase activity, this organism was comparable to superoxide dismutase-containing species in its resistance toward hyperbaric O_2 and toward the oxygen-dependent lethality of plumbagin. *L. plantarum* required Mn-rich media and actively accumulated Mn(II). Soluble extracts were found to contain approximately 9 μg of Mn per mg of protein and 75 to 90% of this Mn was dialyzable. Such extracts exhibited a dialyzable and ethylenediaminetetraacetic acid-inhibitable ability to scavenge $O_2^{\cdot-}$. This $O_2^{\cdot-}$-scavenging activity was due to the dialyzable Mn(II) present in these extracts and could be mimicked by $MnCl_2$. Cells grown in Mn-rich media were enriched in dialyzable Mn and were more resistant toward oxygen toxicity and toward the oxygen-dependent plumbagin toxicity than were cells grown in Mn-deficient media. *L. plantarum* exhibited no nutritional requirement for iron and little or no iron was present in these cells, even when they were grown in iron-rich media. *L. plantarum* thus appears to use millimolar levels of Mn(II) to scavenge $O_2^{\cdot-}$, much as most other organisms use micromolar levels of superoxide dismutases.

ENZYMATIC BASIS OF DETOXICATION VOL I

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Chapter 15

Superoxide Dismutases: Detoxication of a Free Radical

H. MOUSTAFA HASSAN and IRWIN FRIDOVICH

I. INTRODUCTION

Most of the life forms we see are absolutely dependent on molecular oxygen, yet all living cells are prone to oxygen toxicity. The toxicity of oxygen has been related to the intermediates of oxygen reduction including the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$). These are very reactive substances which can directly or indirectly cause substantial damage to living cells. It is obvious, therefore, that the first and the best defense against oxygen toxicity would be avoidance of the generation of these reactive intermediates. Indeed, this is the case and most of the oxygen consumed by respiring cells is used by the cytochrome oxidase system which accomplishes the tetravalent reduction of oxygen to water, without the release of any free reactive intermediates. Nevertheless, both O_2^- and H_2O_2 are normal, if minor, products of the biological reduction of oxygen, and aerobic organisms are able to survive by virtue of a unique set of defensive enzymes that scavenge these reactive intermediates of oxygen reduction. Obligate anaerobes and mutants lacking these enzymes are killed upon exposure to atmospheric oxygen. The superoxide radical is eliminated by the superoxide dismutases which catalyze Eq. (1) while the hydrogen peroxide is removed by catalases and/or peroxidases. This chapter will attempt to summarize our current knowledge on the nature of superoxide dismutases, and on their physiological role in providing protection against oxygen toxicity.

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

Brawn, K., and Fridovich, I. Superoxide radical and superoxide dismutases: threat and defense. Acta Physiol. Scand. Suppl. 492, 9-18 (1980).

BRAWN, K. & FRIDOVICH, I.: Superoxide radical and superoxide dismutases: threat and defense. Acta Physiol Scand 1980, Suppl. 492: 9–18. Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA.

An enzymic flux of O_2^- and H_2O_2 caused strand breaks in the supercoiled covalently closed circular Col E1 plasmid. Subnanomolar levels of superoxide dismutase or of catalase prevented this attack on DNA, signifying that both O_2^- and H_2O_2 were required. Benzoate, mannitol or histidine, which do not scavenge O_2^- or H_2O_2, also protected the DNA suggesting that the proximate attacking species had a reactivity comparable to that of the hydroxyl radical. Replacing EDTA with diethylene triamine pentaacetic acid eliminated this attack. In toto the data suggest a metal-catalyzed interaction between O_2^- and H_2O_2 which generates a potent oxidant, possibly OH·, which can cause DNA strand scission. The biological implications of the production and the enzymic scavenging of the superoxide radical are discussed.

Key words: Superoxide radical, hydrogen peroxide, hydroxyl radical, superoxide dismutase, catalase, methyl viologen, pyocyanine, DNA strand scission

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A Reaction of the Superoxide Radical with Tetrapyrroles[1]

PHILMORE ROBERTSON, JR., AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Bilirubin and biliverdin were bleached during exposure to the aerobic xanthine oxidase reaction. Enzymic scavenging of O_2^-, by superoxide dismutase, inhibited, whereas enzymic scavenging of H_2O_2, by catalase, did not. Increasing the rate of production of O_2^- without increasing the turnover rate of xanthine oxidase, by increasing pO_2, accelerated the bleaching of the biliverdin. Moreover, a scavenger of OH·, such as benzoate, or an inactivating chelating agent for iron, such as diethylenetriamine pentaacetate or desferrioxamine mesylate, did not inhibit. It follows that O_2^- can directly attack these tetrapyrroles. Kinetic competition between superoxide dismutase and bilirubin yielded a value for $k_{\text{bilirubin, O2}} = 2.3 \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}$ at pH 8.3 and at 23°C. A similar experiment for biliverdin yielded a value for $k_{\text{biliverdin, O2}} = 7 \times 10^3\ \text{M}^{-1}\ \text{s}^{-1}$.

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Cyanide Catalyzes the Oxidation of α-Hydroxyaldehydes and Related Compounds: Monitored as the Reduction of Dioxygen, Cytochrome *c*, and Nitroblue Tetrazolium[1]

PHILMORE ROBERTSON, JR., SHARON E. FRIDOVICH, HARA P. MISRA, AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received July 9, 1980

Cyanide catalyzed the oxidation of α-hydroxycarbonyls and of related compounds. In the cases of glyceraldehyde 3-phosphate and of dihydroxyacetone phosphate the tautomeric enediol was the obligatory intermediate which reacted with cyanide yielding the active reductant. Cytochrome *c*, nitroblue tetrazolium, and dioxygen were all reduced by this reductant. In the case of dioxygen the product was the superoxide radical which could then secondarily reduce cytochrome *c* or nitroblue tetrazolium. In air-equilibrated reaction mixtures, at 25°C, approximately 35% of cytochrome *c* reduction and 95% of nitroblue tetrazolium reduction was mediated by superoxide, as judged from susceptibilities to inhibition by superoxide dismutase. Since the oxidations observed were univalent, carbon-centered radicals appear to be necessary intermediates, and their secondary reactions generated a multiplicity of products, seen as smears on thin-layer chromatograms. Free cyanide must be regenerated during these secondary reactions, since cyanide functioned catalytically in the overall process. A partial mechanism has been proposed in explanation of these observations.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
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DNA Strand Scission by Enzymically Generated Oxygen Radicals[1]

KAREN BRAWN AND IRWIN FRIDOVICH[2]

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Col E1 DNA suffers strand scission when exposed to xanthine oxidase acting aerobically on xanthine. Strand scission was prevented by low levels of superoxide dismutase or of catalase. Mannitol, benzoate, or histidine, which scavenge OH but which react with neither O_2^- nor H_2O_2, also prevented strand scission. Replacement of 0.1 mM ethylenediaminetetraacetate by 0.1 mM diethylenetriaminepentaacetate prevented strand scission. Three mechanisms for the production of OH, or of a comparably powerful oxidant, by metal-catalyzed interaction of O_2^- with H_2O_2, are proposed.

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Superoxide dismutases: defence against endogenous superoxide radical

IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

Abstract Attempts to measure the rate of O_2^- production, in whole cells or in intact subcellular organelles, are frustrated by the endogenous superoxide dismutase (SOD). *Streptococcus faecalis* contains a single manganese-SOD which was isolated and used as an antigen in the rabbit. A precipitating and inhibiting antibody was obtained and used to suppress the SOD in crude lysates of *S. faecalis*. It allowed the demonstration that 17% of the total oxygen uptake by such lysates, in the presence of NADH, was associated with O_2^- production.

O_2^- attacks unsaturated lipids and breaches the integrity of membranes. When the membranes are free of lipid hydroperoxides, then both O_2^- and H_2O_2 are required and singlet oxygen appears to be the proximal attacking species. When the membrane contains some lipid hydroperoxide, then O_2^- is itself sufficient and seems to generate an alkoxy radical, by reacting with the lipid hydroperoxide. It appears likely that attack on membranes is one of the reasons for the cytotoxicity of O_2^-.

In *Escherichia coli* the manganese-SOD is derepressed by O_2^-. This enzyme is not made in the absence of oxygen and in aerobic conditions any change which results in enhanced production of O_2^- calls forth an increased synthesis of this enzyme. Increased levels of SOD, however achieved, correlate with greater resistance towards oxygen toxicity.

It is generally true that respiring cells contain more SOD than non-respiring cells. Among obligate anaerobes there is a correlation between SOD-content and tolerance towards oxygen. It is not known whether the SOD in obligate anaerobes is a retained primitive characteristic or one recently acquired by plasmid transfer.

There is an exception to the rule that copper-zinc-SOD is found in eukaryotes but not in prokaryotes, and that is the symbiotic bacterium *Photobacterium leiognathi*. This symbiont may have obtained the Cu-ZnSOD gene from the host fish.

Ilan, Y., Fridovich, I., Pasternak, R. F., and Rabani, J. Superoxide dismuting activity of an iron porphyrin. Inorg. Nucl Chem. Lett. 17, 93 (1981).

Abstract: The efficiency of tetrakis-(4-N-methylpyridyl)porphineiron(III) as a superoxide dismuting catalyst has been determined using a pulse radiolysis technique.

Superoxide radical (O_2^-) is a minor but nontrivial product of biological oxygen reduction and its potential reactivities threaten the integrity of respiring cells. This danger is minimized by superoxide dismutases (SOD) which catalyze the disproportionation of this radical (1-3). Low molecular weight compounds which could mimic the catalytic activity of SOD and which might be able to cross cell membranes would be useful experimental tools. Aquocopper(II) catalyzes this disproportionation (4-5), but chelating agents which abound in cells tend to diminish its catalytic activity. A number of metal complexes that have been reported as catalyzing the disproportionation reaction have since been shown to be inactive (6,7) and several others show only limited activity near neutral pH (8).

Recently a potential mimic of SOD was claimed which might circumvent some of the problems described above (9). This material, tetrakis-(4-N-methylpyridyl)porphineiron(III), Fe(III)TMpyP, is stable, water soluble, and was suggested as being as much as 3% as effective as SOD (9,10). These results were based on an indirect assay method--inhibition of the conversion of nitroblue tetrazolium to formazan--and involved a mathematical model developed for this assay but heretofore untested (9). Pulse radiolysis appeared to be the method of choice for verifying the O_2^- dismuting activity of Fe(III)TMpyP and for probing its mechanism of action. Reported below are results confirming the catalytic activity, and work is in progress which should elucidate the detailed mechanism for this activity.

J. P. Martin, Jr., and I. Fridovich, Evidence for a natural gene transfer from the ponyfish to its bioluminescent bacterial symbiont Photobacter leiognathi. The close relationship between bacteriocuprein and the copper-zinc superoxide dismutase of teleost fishes. J. Biol. Chem. 256, 6080-6089 (1981).

The copper and zinc-containing superoxide dismutases of six teleost fish species and of the bioluminescent bacterium *Photobacter leiognathi* were isolated and characterized, as was the iron-containing superoxide dismutase of *P. leiognathi*.

The amino acid composition of the CuZn enzyme from *P. leiognathi* was more closely related to the corresponding fish enzymes than it was to any other yet described CuZn superoxide dismutase. Rank order correlation analysis of amino acid difference indices, computed for all pairwise comparisons of bacteriocuprein and the ponyfish enzyme with other CuZn-superoxide dismutases, suggested that the bacterial and ponyfish enzymes occupy similar phylogenetic positions, being most closely related to the superoxide dismutases of teleost fish. Discriminant functions analysis of all available superoxide dismutase amino acid compositions demonstrated that correct group classification could be achieved on the sole basis of amino acid composition. Thus, Fe and Mn superoxide dismutase groups could be delineated and both groups differed dramatically from the CuZn-superoxide dismutases. Bacteriocuprein was classified with the CuZn-superoxide dismutase group. Moreover, this analytical method subdivided the CuZn group into three subgroups made up of the avian plus mammalian, plant plus fungal, and the fish enzymes, with the *P. leiognathi* enzyme placed in the fish subgroup. The physicochemical properties of the *P. leiognathi* CuZn-superoxide dismutases were very similar to those of the comparable CuZn enzymes from other sources, especially those of teleost fish.

In view of the long standing symbiosis between *P. leiognathi* and its teleost hosts, the leiognathid fishes, these results suggest that the gene specifying the CuZn-superoxide dismutase was transferred from the fish to the bacterium, during their coevolutionary history.

Archibald, F. S., and Fridovich, I. Manganese, superoxide dismutase and oxygen tolerance in some lactic acid bacteria. J. Bacteriol. 146, 928-936 (1981).

A previous study of the aerotolerant bacterium *Lactobacillus plantarum*, which lacks superoxide dismutase (SOD), demonstrated that it possesses a novel substitute for this defensive enzyme. Thus, *L. plantarum* contains 20 to 25 mM Mn(II), in a dialyzable form, which is able to scavenge O_2^- apparently as effectively as do the micromolar levels of SOD present in most other organisms. This report describes a survey of the lactic acid bacteria. The substitution of millimolar levels of Mn(II) for micromolar levels of SOD is a common occurrence in this group of microorganisms, which contained either SOD or high levels of Mn(II), but not both. Two strains were found which had neither high levels of Mn(II) nor SOD, and they were, as was expected, very oxygen intolerant. Lactic acid bacteria containing SOD grew better aerobically than anaerobically, whereas the organisms containing Mn(II) in place of SOD showed aerobic growth which was, at best, equal to anaerobic growth. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) increases the rate of O_2^- production in these organisms. *Lactobacillus* strains containing high intracelluar Mn(II) were more resistant to the oxygen-dependent toxicity of plumbagin than were strains containing lower levels of Mn(II). The results support the conclusion that a high internal level of Mn(II) provides these organisms with an important defense against endogenous O_2^-.

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Isolation and Characterization of the Iron-Containing Superoxide Dismutase of *Methanobacterium bryantii*[1]

THOMAS W. KIRBY, JACK R. LANCASTER, JR.,[2] AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Methanobacterium bryantii contains a single electrophoretically discernible superoxide dismutase, which constitutes 0.4% of the extractable protein. This enzyme has been purified to electrophoretic and ultracentrifugal homogeneity. It appears to be a tetramer. The subunits were tenaciously, but noncovalently bonded and were of identical size. The molecular weight of the enzyme was found to be 91,000 ± 2000. The specific activity of this enzyme was identical to that previously noted for the corresponding enzyme from *Escherichia coli*. The enzyme contained 2.7 atoms of Fe, 1.7 atoms of Zn, and less than 0.2 atoms Mn per tetramer. Its amino acid composition placed this enzyme with the other Mn- and Fe-containing superoxide dismutases. The *M. bryantii* enzyme was also similar to previously described Fe-containing superoxide dismutases in its optical and electron paramagnetic resonance spectra and in its susceptibility to inactivation by H_2O_2. The *M. bryantii* enzyme was ininhibited by N_3^-, but was less sensitive towards this inhibitor than other iron-containing superoxide dismutases.

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The Scavenging of Superoxide Radical by Manganous Complexes: *In Vitro*[1]

FREDERICK S. ARCHIBALD AND IRWIN FRIDOVICH[2]

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received September 9, 1981

Dialyzable manganese has been shown to be present in millimolar concentrations within cells of *Lactobacillus plantarum* and related lactic acid bacteria. This unusual accumulation of Mn appears to serve the same function as superoxide dismutase (SOD), conferring hyperbaric oxygen and superoxide tolerance on these SOD-free organisms. The form of the Mn in the lactic acid bacteria and the mechanisms whereby it protects the cell from oxygen damage are unknown. This report examines the mechanisms by which Mn catalytically scavenges O_2^-, both in the xanthine oxidase/cytochrome c SOD assay and in a number of *in vitro* systems relevant to the *in vivo* situation. In all the reaction mixtures examined, Mn(II) is first oxidized by O_2^- to Mn(III), and H_2O_2 is formed. In pyrophosphate buffer the Mn(III) thus formed is re-reduced to Mn(II) by a second O_2^-, making the reaction a true metal-catalyzed dismutation like that catalyzed by SOD. Alternatively, if the reaction takes place in orthophosphate or a number of other buffers, the Mn(III) is preferentially reduced largely by reductants other than O_2^-, such as thiols, urate, hydroquinone, or H_2O_2. H_2O_2, a common product of the lactic acid bacteria, reacted rapidly with Mn(III) to form O_2, apparently without intermediate O_2^- release. Free hexaquo Mn(II) ions were shown by electron spin resonance spectroscopy and activity assays in noncomplexing buffers to be poorly reactive with O_2^-. In contrast, Mn(II) formed complexes having a high catalytic activity in scavenging O_2^- with a number of organic acids, including malate, pyruvate, propionate, succinate, and lactate, with the Mn-lactate complex showing the greatest activity.

Investigations of the State of the Manganese in Lactobacillus Plantarum. F. S. Archibald and I. Fridovich, Arch. Biochem. Biophys., in press.

Abstract

The Mn(II) in Lactobacillus plantarum, which constitutes a functional replacement for superoxide dismutase, is primarily associated with a complex large enough to be retained by a 0.45 μ-cutoff ultrafilter. This ligand retards, but does not entirely prevent, dialysis of the Mn(II) from cell extracts. EDTA, but not pyrophosphate, rapidly displaces this endogenous ligand. Streptococcus faecalis, which unlike L. plantarum does not accumulate large amounts of Mn(II) does not contain a large Mn(II)-restraining ligand. L. plantarum accumulates approximately 60 mM phosphate as high molecular weight polyphosphate. There are thus approximately two residues of phosphate, as non-dialyzable polyphosphate, per Mn(II) in these cells. Growth in phosphate-deficient medium decreases the cell content of polyphosphate and prevents accumulation of Mn(II). Polyphosphate does bind Mn(II) and Mn(II)-polyphosphate exhibited an O_2^- dismuting activity which was similar to that of the Mn(II) in cell extracts. The ESR spectrum of Mn(II)-polyphosphate, in the presence of orthophosphate and NaCl, approximated that of cell extracts. Proteolytic digestion of cell extracts decreased the apparent size of the Mn(II)-ligand, allowing it to pass a 0.2 μ filter. It thus appears likely that the Mn(II) in L. plantarum is associated with polyphosphate-protein aggregates.

Superoxide radical inhibits catalase.
Y. Kono and I. Fridovich, J. Biol. Chem., submitted.

Summary

Catalase was inhibited by a flux of O_2^- generated *in situ* by the aerobic xanthine oxidase reaction. Two distinct types of inhibition could be distinguished. One of these was rapidly established and could be as rapidly reversed by the addition of superoxide dismutase. The second developed slowly and was reversed by ethanol, but not by superoxide dismutase. The rapid inhibition was probably due to conversion of catalase to the ferro-oxy state (compound III), while the slow inhibition was due to conversion to the ferryl state (compound II). Since neither compound III nor compound II occurs in the catalatic reaction pathway, they are inactive. This inhibition of catalase by O_2^- provides the basis for a synergism between superoxide dismutase and catalase. Such synergisms have been observed *in vitro* and may be significant *in vivo*.

Summary

Streptococcus sanguis, whose growth appears to be independent of the availability of iron, makes no hemes, contains neither catalase nor peroxidase, and can accumulate mM levels of H_2O_2 during aerobic growth. It possesses a single manganese-containing superoxide dismutase whose concentration can be varied over a 50-100 fold range by manipulating the availability of oxygen during growth. Cell extracts contain a soluble NADH-plumbagin diaphorase which mediates O_2^- production in vitro and presumably also in vivo. Plumbagin increased oxygen consumption by S. sanguis and imposed an oxygen-dependent toxicity. Cells grown aerobically and containing elevated levels of superoxide dismutase were resistant to this toxicity. DMSO, which was shown to freely permeate S. sanguis, was used as an indicating scavenger of OH·. An in vitro enzymic source of O_2^- plus H_2O_2 generated formaldehyde from DMSO, an indication of OH· production. Either superoxide dismutase or catalase inhibited this OH· production and iron salts augmented it. Intact, aerobic, cells of S. sanguis also gave evidence of OH· production, in the presence of plumbagin, but all of it appeared to be generated outside the cells. In addition 0.5 M DMSO did not diminish the oxygen-dependent toxicity of plumbagin. We conclude that, in S. sanguis, O_2^- can exert a toxic effect independent of the production of OH·.